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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Superantigens (SAGs) are a group of immunostimulatory and disease associated proteins of bacterial or viral origin that bind to MHC molecules and certain T cell receptor (TCR) Vb domains and can result in systemic shock and death. Rational design of strategies for prevention or treatments of such diseases may only be possible if we have insights into the mechanisms of T cell activation by SEs. During this grant, we cloned and expressed a human TCR gene in bacculovirus expression system. We demonstrated that the expressed TCR was properly folded and was functional. We also produced soluble human MHC class II HLA-DR1 and studied the kinetics of its interactions with TCR and different Superantigens by several biochemical and biophysical methods. We measured the kinetics of interactions between SAGs and HLA-DR1 and the formation of ternary and quaternary complexes between TCR, DR1, peptides, and SAGs. We discovered that SAGs such as SEA and SEH bind HLA-DR1 or HLA-DR1 in complex with peptide and enforce rigidity in the groove of MHC II that prevents conformational changes necessary for association with a new peptide, or dissociation of the bound peptide. These observations change the conventional understanding of SAGs/MHC by TCR and helps in clever designs of therapeutics.

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Final Report for DAMD17-00-1-0671, August 2000-March 2004
Scheherazade Sadegh-Nasseri, PhD., Principal Investigator

INTRODUCTION

Superantigens (SAGs) are a group of immunostimulatory and disease associated proteins of bacterial or viral origin that bind to MHC molecules and certain TCR Vβ domains. Staphylococcal enterotoxins, (SEs), such as SEB, bind as folded proteins to both major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells and germline-encoded variable domain sequences of specific T cell receptor (TCR) beta chain on T lymphocytes. SAGs stimulate at nano to picogram concentrations up to 20-30% of human T cell repertoire, resulting in the massive release of T cell derived cytokines such as interleukin (IL-2) and tumor necrosis factor (TNF)β. Consequently, staphylococcal enterotoxins are potential biological threat agents. Exposure to SEs can result in systemic shock and death of military personnel. Rational design of strategies for prevention (vaccines) or treatments of such diseases may only be possible if we have insights into the mechanisms of T cell activation by enterotoxins. Thus, our efforts focused on cloning and production of a TCR protein and biochemical characterization of its interaction with its natural ligands, MHC/peptide, and enterotoxins. During this grant, we have accomplished all the goals proposed in the application. We cloned and expressed a human T cell receptor gene in bacculovirus expression system. We demonstrated the proper folding and functionality of the protein. We produced soluble human HLA-DR1 and HLA-DM and studied the kinetics of interactions of the TCR with HLA-DR1 and different superantigens by several biochemical and biophysical methods. We measured the kinetics of interactions between SAGs and HILA-DR1 and the formation of ternary and quaternary complexes between TCR, DR1, peptides, and SAgs. Our investigations unraveled new concepts in molecular recognition of peptide/MHC and SAgs by TCR. We discovered that SAGs such as SEA and SEH and not SEB or TSST-1, bind HLA-DR1 or HLA-DR1 in complex with peptide and enforce rigidity in the groove of MHC II that prevents conformational changes necessary for opening of the groove for association with a new peptide. Likewise, dissociation of the bound peptide was inhibited. Both association and dissociation of peptides were inhibited even in the presence of HLA-DM. As promised, recombinant bacculovirus containing the TCR genes is ready for transfer to the USAMRIID

RESULTS

CL-1 T cell responses to SAGs

CL-1 T cells are one of the very rare human T cell clones that have been propagated in tissue culture for an extended period of time. CL-1 cells are specific for $HA_{306-318}$ peptide of influenza hemagglutinin ATexas77. Our interest in this T cell clone stems from extensive work on HLA-DR1 and $HA_{306-318}$ peptide interaction and the TCR recognition of these complexes (Korb et al., 1999; Mirshahidi et al., 2004; Mirshahidi et al., 2001). In an initial examination of SAG reactivity with CL-1 T cells, we found that complexes of SEC2 bound to DR1 were stimulatory to the CL-1 T Cells as shown in Fig 1. In contrast, SEH and SEA binding to HLA-DR1 were inhibitory to the recognition of the specific $HA_{306-318}$ peptide bound in the DR1 groove (see explanation below). At the highest doses of peptide tested, 1mM, additional response to SEC-2 was not observed for unknown reasons to us at this time. Confirming data were generated using cloned and purified

TCR from CL-1 as discussed later. Therefore, CL-1 TCR can be studied for understanding of the molecular mechanisms of SEC-2 toxicity and design of reagents that can inhibit its toxicity.

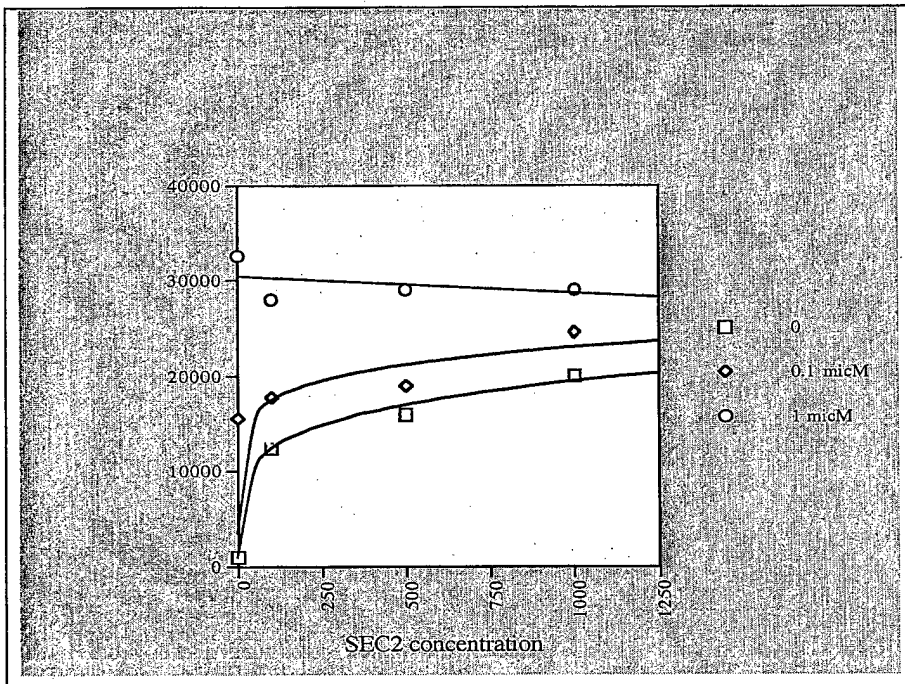

Figure 1. Cl-1 T cells proliferate to SEC-2.

Clone-1 T cells were stimulated by DR1 expressing EBV transformed B cells, expressing HLA-DR1, which were pre-pulsed with 0, 0.1, or 1 μM $HA_{306-318}$ and washed. Different amounts of SEC-2 as shown on the x-axis were added throughout the four-day culture. T Cells were tested for proliferation after 4 days of stimulation.

Cloning and expression of CL-1 TCR

Strategies and procedures

A. Cloning and expression of soluble human T cell receptor for in vitro biophysical studies

Production of TCR protein has been a long challenge over the past decade. For some unknown reasons, production of soluble TCR has successfully been possible only for very few clonotypes. Therefore, we attempted undertaking our efforts towards this goal bearing in mind that we might have to tackle multiple strategies. The general protein design incorporated addition of Acidic and basic chains of Leucine Zipper to α and β TCR accordingly. To ease protein-protein interactions we also designed an unpaired Cys at the c-terminus of the β chain. The design strategy is shown in Fig 3A. This design was maintained for both E. *coli.* and Bacculovirus expression systems.

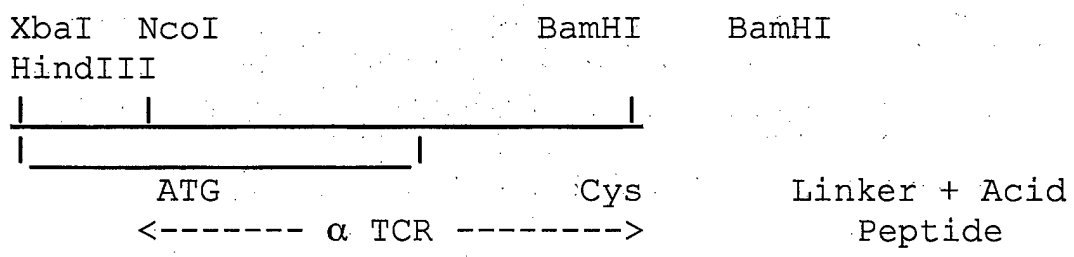

Figure 2. TCR PCR and the Fusion with Linker-Zipper

i. Bacterial expression system

In the first effort, we tried a bacterial expression system, because of the possibility of getting large quantities of folded protein once all the technical issues were successfully addressed. Two individual constructs for bacterial expression of α and β TCR as leucine-zipper fusion proteins were designed. The leucine zipper used here is based on the modified sequence of the naturally occurring leucine-zipper region of Fos and Jun oncoproteins (O'Shea et al., 1989). The α construct, α-ALZ, consist of the genes encoding the α-chain variable and constant domains of Clone1 TCR fused via a linker to the acidic peptide of the leucine zipper. The β-construct, β-BLZ, is made up of the genes encoding the β-chain variable and constant domains of Clone1 TCR fused, through a linker, to the basic peptide of the leucine zipper. In addition, the β-construct also has a free unpaired cysteine after the last residue of the basic leucine zipper peptide to facilitate specific immobilization (for BIAcore studies) and fluorescent labeling. The TCR sequences were included up to the cysteine residues involved in the interchain disulfide bridge at the C-terminus of constant domains. A cysteine residue was added to the c-terminus of β chain to facilitate immobilization of TCR in proper orientation for biophysical studies.

The cDNA of α and βT-Cell receptor from Clone 1 T-Cells were constructed using RT-PCR. From the cDNA, the individual constructs for α and β TCR for bacterial expression were made using PCR amplification as follows. The 5' primers were designed to introduce an initiating methionine codon, as part of an NcoI site, just before the first residue of the mature α and β subunits as well as to introduce an XbaI site upstream of NcoI site for cloning purposes. The 3' primers were designed to exclude all the residues following the interchain disulfide as well as to introduce a BamHI site immediately following this cysteine residue. Both such engineered α and β-TCR were cloned into Pbluescript II KS vector as XbaI–BamHI fragments.

The DNA coding for the acid/basic leucine zipper and the linker (GSTTAPS) connecting the zipper to the TCR subunits were made using six synthetic oligonulceotides. Briefly, six primers (3 each for the sense and the anti-sense strands), designed to make cohesive BamH1 and HindIII sites on either side, were melted and annealed. These annealed double-stranded DNA, coding for either the acid-LZ peptide linker or the basic-LZ peptide linker, were then ligated

following the BamHI end of pBluescript construct carrying either the α- or β-TCR. Codon usage in *E. coli* was used as a basis for constructing the DNA sequence coding for the leucine-zipper. Furthermore, the unpaired cysteine in the constant domain of β-TCR was mutated to Alanine, to enable more efficient refolding, using Quik Change Mutagenesis technique (Stratagene). The α-ALZ and β-BLZ clones were entirely sequenced to ensure the coding regions had the intended sequence. The NcoI –HindIII fragments of α-ALZ and β-BLZ constructs were then cloned in to pET21-d and pET23-d (Novagen) expression vectors bearing the T7 polymerase promoter. pET21-d has a T7lac promoter contains a lac operator sequence downstream of T7, offers a more stringent control over uninduced basal protein expression, and is typically used to express proteins that may be toxic to bacteria. pET23-d has a plain T7 promoter and works very well with not-so toxic proteins.

Protein expression was attempted in two different bacterial strains – BL21(DE3) pLys S and BL21(DE3)-CodonPlus-RIL (Stratagene). BL21(DE3)-CodonPlus-RIL (cells contain extra copies of the genes that code for t-RNAs (Arginine, Isoleucine and Leucine), which most frequently limit translation of heterologous proteins in E. coli.

Four possible combinations in terms of expression vectors and hosts were tried. They were:
1) pET21d- based plasmid in BL21(DE3) pLys S cells
2) pET23d- based plasmid in BL21(DE3) pLys S cells
3) pET21d- based plasmid in BL21(DE3)-CodonPlus-RIL cells.
4) pET23- based plasmid in BL21(DE3)-CodonPlus-RIL cells.

As for α goes, there was very little expression observed with BL21(DE3) pLys S cells both with pET21d and pET23d vectors. noticeable expression was observed with BL21(DE3)-CodonPlus-RIL cells both with pET21d and pET23d vectors. The combination that worked the best so far, in terms of the size of the inclusion body like pellets, is pET21d-α-ALZ plasmid in BL21(DE3)-CodonPlus-RIL cells. As for the β chain goes, both cell lines and both vectors worked very well. Out of the possible combinations, pET21d-mut β-BLZ plasmid in BL21 (DE3) pLys S and BL21(DE3)-CodonPlus-RIL cells work very well.

The proteins were expressed individually as inclusion bodies in the bacterial strain, BL21(DE3)-CodonPlus-RIL (Stratagene). Inclusion bodies were purified and solubilized in either urea or guanidine. The α- and β- chains were renatured together by dilution refolding of the denatured protein to form heterodimeric TCR. We followed the refolding procedure described by Garboczi et al first. Later on, we modified his procedure for optimization of refolding.

Beta chain was expressed in inclusion bodies at or > 90% purity, as evaluated by SDS-PAGE and Coomasie staining. However, alpha chain expression was poor and in mixture with many other proteins. Changing the strain of expressing E. coli helped to increase the proportion of the α chain relative to other proteins but did not eliminate expression of contaminating proteins significantly.

Small-scale refolding tests were set up to evaluate efficiency of the refolding conditions. We tested different denaturants, changes in pH, presence, or absence of glycerol at varying concentrations (0-30 %), and different ratios of the denatured individual proteins. We continue

our efforts in evaluating refolding conditions to find the optimal conditions for TCR folding and large-scale purification.
Parallel with our protein purification and refolding efforts, we have been working towards establishing the technology needed for accomplishing specific Aim 2. A novel membrane immobilization strategy for investigating interactions of TCR inserted in cell membrane with the ligand in real time was attempted. Membrane fragments of T cell clones bearing TCR and other proteins involved in T cell activation, such as CD4, were isolated and used for immobilization on the surface of L1 chip of BIAcore. This technique can be utilized for measuring interaction of TCR inserted in membranes with Superantigens or peptide/MHC ligands in natural environment. Purified TCR immobilized on the surface of BIAcore chip will be compared to the membrane anchored native TCR for accurate determination of the mechanism of interaction. Possibilities of TCR clustering in interaction with MHC/peptide and different Superantigens will be evaluated.

We found that we can properly fold TCR from bacteria, as confirmed by ELISA, but with a low yield. We attempted to optimize refolding of TCR by; **a)** mixing a larger proportion of a chain protein with the beta chain, **b)** try several protein folding methods, and **c)** use other strains of bacteria expressing the gene to increase compatibility with the a chain. In addition, we tried adding different concentrations of glycerol (known to improve proper folding of denatured proteins) to the folding mixture. Five percent glycerol helped refolding slightly, whereas other concentrations of glycerol up to 50% did not lead to improved folding over the glycerol free folding mixture.

Unfortunately, our tedious efforts in obtaining high yields of correctly folded bacterial produced TCR$\alpha\beta$ did not lead to successful production. Although we detected a small amount of TCR that were identifiable by the anti TCR antibodies specific for the native protein, much of the folded protein was incorrectly folded.

Summary of the TCR cloning in bacterial expression system;

i. Beta chain of TCR was produced in inclusion bodies of the expressing bacteria to over 90 % purity

ii. Alpha chain of TCR was produced in inclusion bodies but mixed with many other proteins

iii. We properly folded TCR from bacteria and confirmed its conformation by Ab recognition in an ELISA. Unfortunately, yield was quite low. We attempted to optimize refolding of TCR by; a) mixing a larger proportion of a chain protein with the beta chain, b) try several protein folding methods, and c) use other strains of bacteria expressing the gene to increase compatibility with the alpha chain. Adding 5% glycerol helped refolding but not high enough to ensure sufficient quantities for large number of experiments.

The constructs for CL-1 TCR expression in E. coli are ready for transfer to the DoD, as part of our agreement.

B- Bacculovirus expression system;

Genes for TCR α- and β- chains were fused to into bacculovirus double promoter vector. The vector was then used for transfection of bacculovirus and the recombinant virus was passed in SF9 cells to increase the virus titer. The supernatant of SF9 cells was collected and used for infecting Hy-5 cells for production of correctly folded CL-1 TCR protein. We were able to successfully express soluble TCR by this method. Supernatant containing CL-1 TCR was passed over an anti-TCR affinity

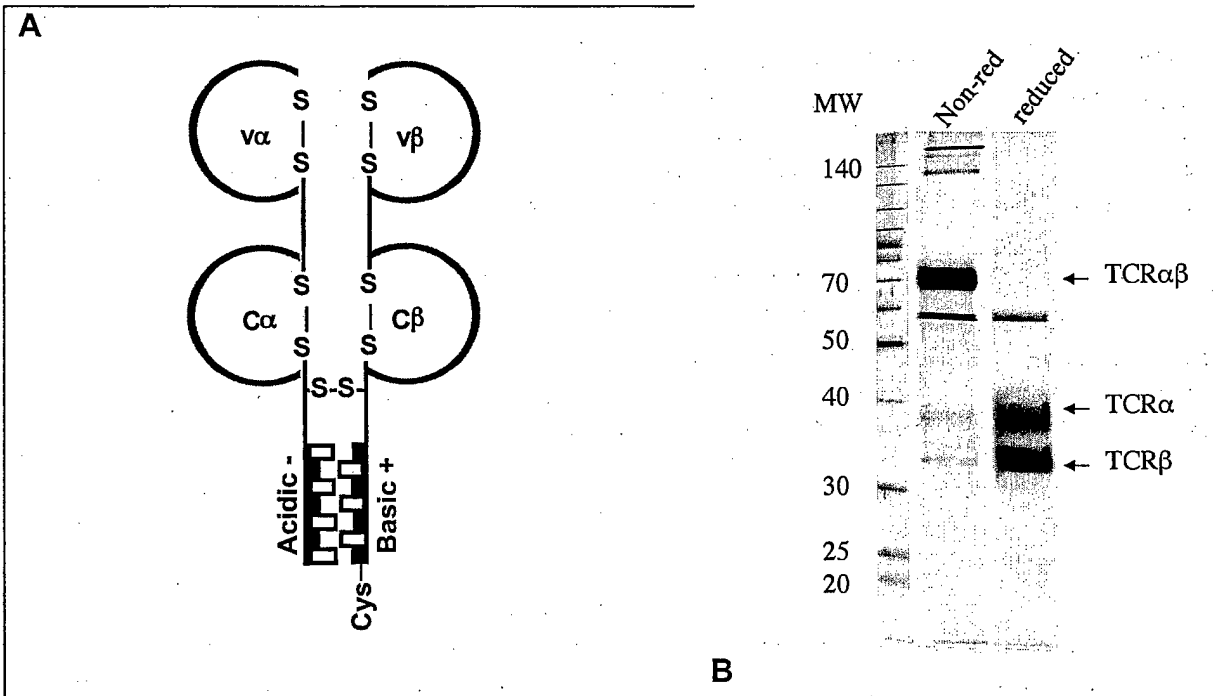

Figure 3. Cl-1 TCR design and purified protein under reducing and non-reducing conditions. A sample of affinity purified Bacculovirus expressed recombinant FLAG-MIC-LZ-TCR was applied to SDS-PAGE and stained using Silver Staining procedure show 90-95 purity. A sharp band at ~60 kDa is a background band.

column and eluted by a high pH buffer (CAP, pH 11.5). We normally obtain an average yield of 1-2 mg/L supernatant per preparation. For affinity purification, we first tried two old frozen hybridoma stocks (12 years past freeze time) from ATCC that were presumably reactive with TCR Vα1.3. Unfortunately, after weeks of fruitless efforts in reviving the frozen cells, we had to request them from a colleague (Dr. Kai Wucherpfennig at Dana Farber Institute at Harvard, MA) who had them in healthy condition. Thus, the hybridoma supernatant was collected and purified over a Protein G column and used for preparation of an affinity column for the TCR purification from the insect cell supernatant.

Biochemical characterization of TCR

Structural characterization of the TCR protein was done using size exclusion and affinity chromatography, polyacrylamide gel electrophoresis (native and denaturing), and antibody reactivity. For determination of the TCR reactivity with its natural ligands, we have used Surface Plasmon Resonance, SPR, and Native-PAGE. TCR reactivity with natural ligands, HLA-DR1 that was either empty, or in complex with different peptides. Additionally, we determined reactivity with different Superantigens including, SEA, SEB, SEH, and SEC2 using SPR.

We routinely use Silver Stain, which has 10-100 folds higher sensitivity than the commonly used Coomassie Stain for protein staining. As such, the purity of the TCR preparations appear to exceed 90% as determined by migration of the α-and β- TCR bands as expected MW in SDS-PAGE under denaturing conditions. A typical SDS-PAGE experiment is depicted in **Fig. 3B**.

The recombinant baculovirus for CL-1 TCR expression is ready for transfer to the DoD as originally planned.

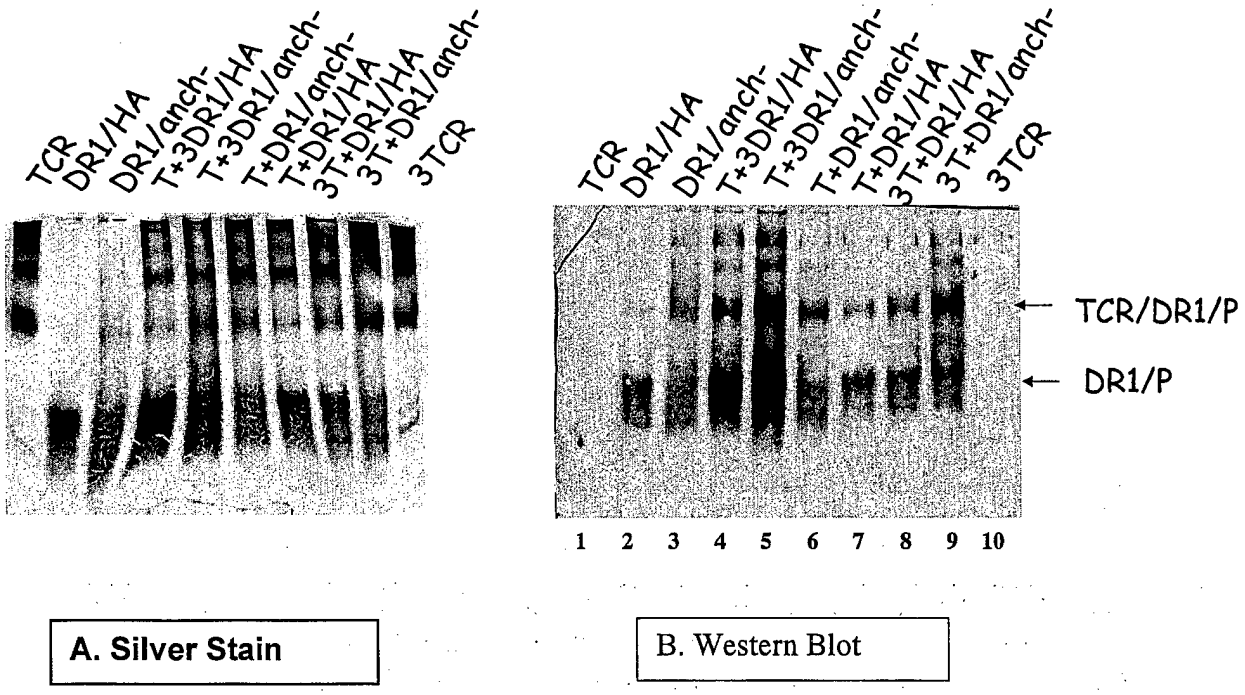

Figure 4. Formation of TCR/DR1/P ternary complex is detected by Western Blot but not by Silver Staining. Silver staining (A) of TCR, DR1/HA306-318, DR1/HA_{anch-}, and TCR + DR1 complex at 1:1, 3:1, or 1:3 ratios fail to identify distinct ternary complex bands in a Native gel because of co-migration with TCR alone. Ternary complexes of purified TCR/DR1/P can be detected by a rabbit polyclonal antibody specific for purified soluble DR1 (Sadegh-Nasseri 1996). Note that this antibody does not react with purified TCR (lanes 1 and 10) although it detects the ternary complexes that migrate as slower migrating species in the native gel. Representative of 3-5 independent experiments.

D. TCR reactivity with DR1

There has been no report of the reactivity of CL-1 TCR with its natural ligand, $DR1/_{HA306-318}$, and HA variant peptides. In previous studies, we have shown that Clone-1 T cells are activated in response to HLA-DR1 expressing B cells pulsed with $HA_{306-318}$ (Korb et al., 1999; Mirshahidi et al., 2001). Thus, in our characterization experiments for CL-1 TCR, we incubated different

concentrations of TCR with variable amounts of preformed DR1/ $HA_{306-318}$ or HA peptide variants that would form either short-lived complexes with DR1, or act as antagonists in T cell stimulation assays. Since all the previous studies with other recombinant TCR proteins have documented low affinity binding between TCR and MHC/peptides, our efforts on demonstration of TCR reactivity with its ligands and formation of ternary complexes focused on detection methods such as using native PAGE and Surface Plasmon Resonance BIACore2000 (Garboczi et al., 1996) suitable for detection of low affinity interactions.

Figure 4 demonstrates two native PAGE gels. In A, the gel is Silver Stained, and in **3B**, a Western Blot of an equivalent gel stained with anti-DR1 antibody is depicted. As shown, although the individual proteins are clearly visible by Silver Stain, presence of distinct ternary complex cannot be detected in A. Silver staining (**Fig. 4A**) of TCR, DR1/HA306-318, DR1/HA_{anch-}, and TCR + DR1 complex at 1:1, 3:1, or 1:3 ratios failed to identify distinct ternary complex bands in a Native gel because of co-migration with TCR alone. I will explain **Fig 4B** later.

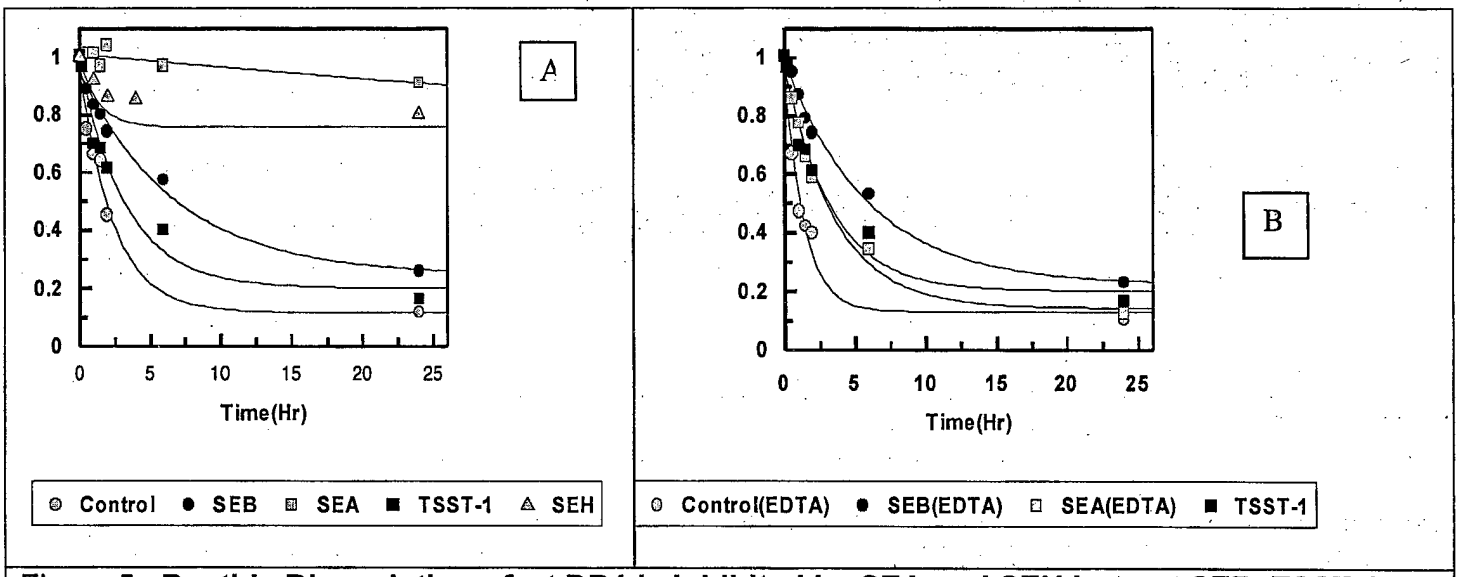

Figure 5. **Peptide Dissociation of wt DR1 is inhibited by SEA and SEH but not SEB, TSST-1.** 2.4 μM wtDR1 in complex with FITC-labeled $HA_{Anchorless}$ was produced and separated. The labeled complexes were dissociated in the presence of 100 times molar excess of relevant unlabeled peptides at 37°C for various times in the absence (○) or presence of 7μM SEA(_), SEA mutant(△), SEB (•), or TSST-1 (■). The dissociation was performed without (A) or with (B) 50 mM EDATA in the buffer. The fluorescence of the labeled complex before dissociation (f_0) is arbitrarily assigned a value of 1.0. The fluorescence of the labeled complex after dissociation for various times is expressed as a percentage of f_0. In the absence of 50 mM EDTA, the dissociation data are fitted to a single exponential curve, which yields $t_{1/2}$ of 1.7 hr for control, 100 hr for SEA, 4.6 hr for SEB, 2.3 hr for TSST-1, and 2.4 hr for SEA mutant. In the presence of 50 mM EDTA, single exponential curve fit *yields* $t_{1/2}$ of 0.94hr for the control, 2.4 hr for SEA, 4 hr for SEB, 1.9 hr for TSST-1, and 2.1hr for SEA mutant. The y- axis represents arbitrary fluorescence units.

To further investigate the interactions of the purified TCR with SAgs bound to MHC II molecules we first had to understand the reactivity of different SAgs with the MHC. The following sections summarize those efforts.

Interaction of SAGs with HLA-DR1

Several SAGs binds to HLA-DR molecules through two distinct binding sites: a low-affinity site ($K_D \sim 10^{-5}$ M) on the conserved α chain and a zinc dependent, high affinity site ($K_D \sim 10^{-7}$ M) on the polymorphic β chain. Certain SAGs bind exclusively to the low affinity site (e.g. Staphylococcal Enterotoxins B (SEB), toxin shock syndrome toxin-1 (TSST-1), whereas others bind to only the high affinity one (e.g. SpeC and SEH), or bind to both the low affinity and high affinity sites (e.g. SEA). The crystal structures of SEB and TSST-1 in complexes with MHC class II molecules have been defined. SEB binds primarily on the N-terminus part of the peptide-binding groove, whereas TSST-1 extends over nearly half the binding groove. Although no crystal structure is available for SEA in complex with MHC class II molecules, mutagenesis and binding studies have shown that SEA binds to class II molecules through two distinct yet cooperative binding sites: a SEB like low affinity site and a high affinity site coordinated by zinc ion with His81β on the MHC class II molecules. Because of two binding sites for class II binding, SEA has been hypothesized to cross-link two MHC molecules.

To address this question we reasoned that for SEA to bind a single DR1 at both low and high affinity binding sites, it would have to bridge over the binding groove of DR1 and interfere with **a)** dissociation of the bound peptide, and **b)** interfere with the specific T cell recognition of peptide/MHC. However, if the binding mode is by cross-linking, effects on peptide dissociation and specific T cell recognition should be minimal.

To test the hypothesis, the dissociation rate of FITC-labeled peptide from wt DR1 complexes was determined in the presence of SEA, SEA mutant, SEB, and TSST-1 at 37 °C in PBS. We observed that the presence of SEA dramatically increased the half life of the wt DR1/ FITC–$HA_{Anchorless}$ from 1.7 hrs to 100 hr in solution and whereas, SEB, SEA mutant, OR TSST-1 had no significant effects on the dissociation rate for FITC–$HA_{Anchorless}$ from wtDR1 molecules (**Fig.5**). Addition of EDTA reversed this effect suggesting that the Zinc dependent binding site is involved.

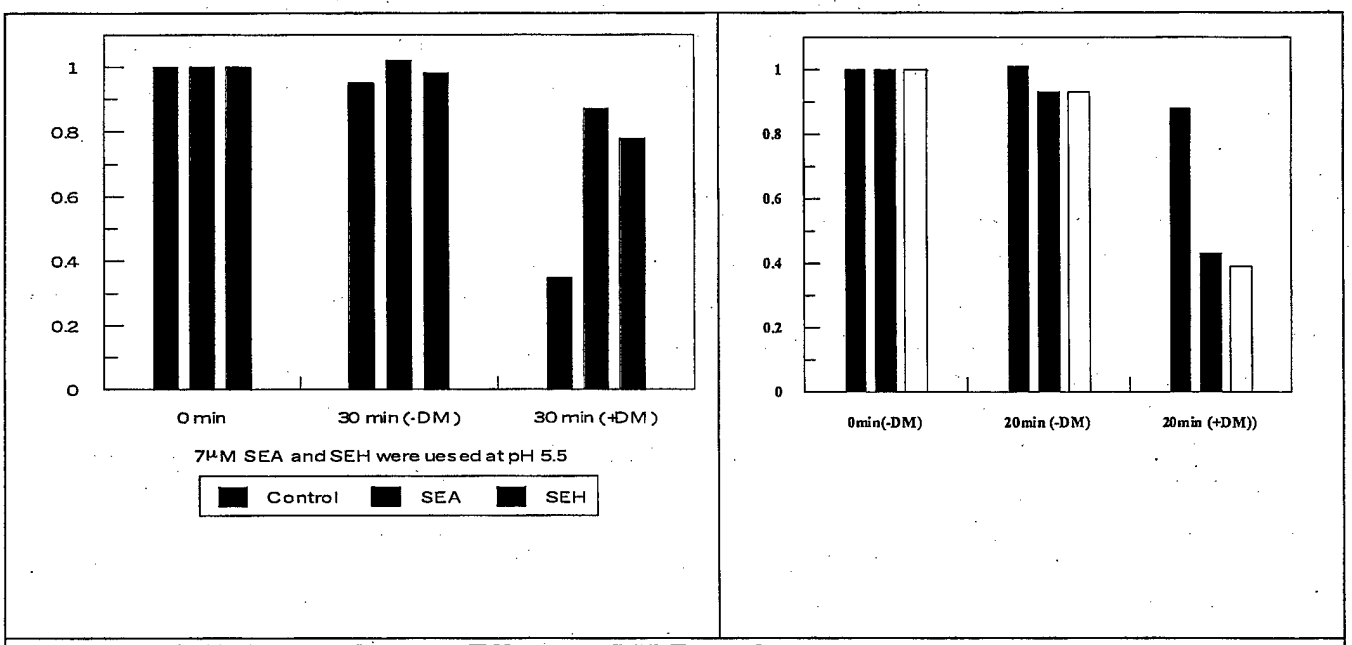

Figure 6. SEA Mutant has no Effect on DM Function

2.4 μM wtDR1 in complex with FITC–$HA_{Anchorless}$ was incubated with 7 μM SEA (solid bar), SEB (shaded bar) or SEA mutant (open bar) in the absence or presence of 1 μM DM (citrate buffer, pH 6.0) for 20 min and dissociation of the complexes was measured.

SEA Mutant has no Effect on DM Function

To determine if the zinc mediated binding site is important for the SEA blocking effect of DM, a mutant SEA, which only binds DR1 at the conserved α domain (LeClaire et al., 2002) was tested. In the absence of DM, upon a 20-min incubation (Fig 6), only 10–15% of wtDR1/FITC-$HA_{Anchorless}$ complexes dissociated in the presence of SEB or mutant SEA. Incubation with DM dissociated over 60% of the complexes during that time period. In contrast, DM did not affect dissociation of wt DR1/ FITC-$HA_{Anchorless}$ complexes in the presence of SEA. We interpreted the data to mean that the Zinc mediated binding site for SEA is required for the blocking phenomena.

SEA, but Not SEB and TSST-1 Blocks the Peptide Binding of DR1 Assisted by DM in Real Time

Recent studies have identified a lateral face that is around pocket 1 of DR1 to be the interaction site between HLA-DM and DR(Chou and Sadegh-Nasseri, 2000). SEA binds to two independent sites on MHC class II molecules. One is on the conserved α chain and the other is through Zinc mediate binding at Hβ81 that is also located on the helix close to pocket 1.

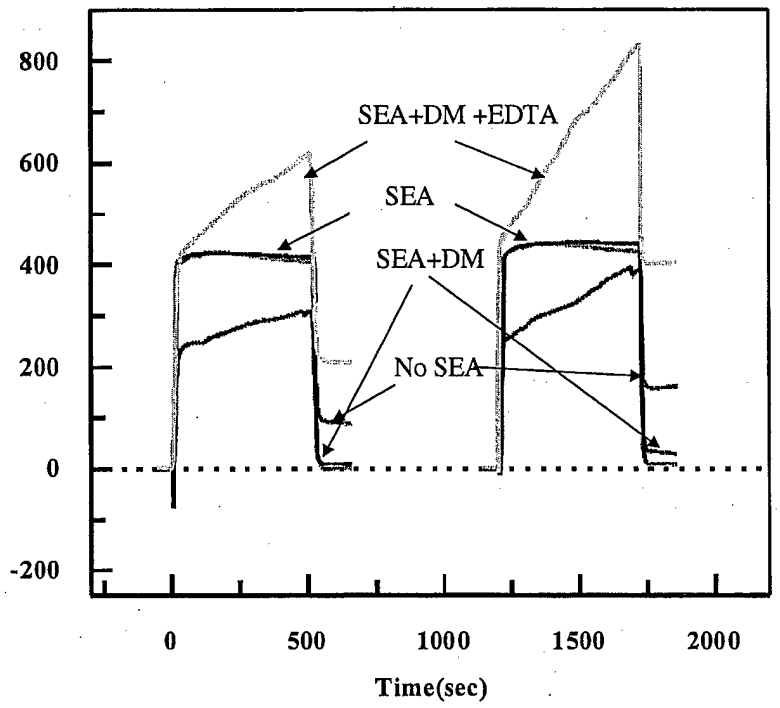

Figure 7. The effect of SEA on the DM assisted peptide binding for wt DR1 in real time.

2.4 μM wtDR1 in complex with HA_{Y308A} peptide was produced and isolated, and then was incubated at 37°C with or without 1 μM DM or 7 μM SEA or both for 20 min. Samples (pH 6.0) were injected over two different peptide surfaces (Cys-HA, and Cys-$HA_{Anchorless}$) at a flow rate of 4 μl/min for 7 min followed by washout at a flow rate of 5 μl/min. Sensograms are shown in different colors: in the presence of DM (blue), in the presence of SEA (red), in the presence of both DM and SEA (black), and DM plus SEA in presence of EDTA (green).

To investigate if SEA can block the DM function, we used a peptide-binding assay by BIAcore surface plasmon resonance (SPR) (Chou and Sadegh-Nasseri, 2000). The peptide surfaces were generated first by Cys coupling to the CM5 chip. To examine formation of DR1–peptide complexes in real time, HA305–318 and $HA_{Anchorless}$ were conjugated to different surfaces of a CM5 chip. Wt DR1 was shaped to be peptide-receptive by incubating with short-lived HA_{Y308A} peptide before it was injected over the surface and binding was monitored. Figure 7 shows binding of wtDR1 to $HA_{Anchorless}$ and $HA_{305–318}$ surfaces in the presence and the absence of DM, SEA, or both. Binding was significantly higher if DM was preincubated with wtDR1 and was present in the sample mix during the injection. If SEA was preincubated with wtDR1 and was present in the sample mix during the injection, no binding was observed. Interestingly, if both DM and SEA were present in the sample mix during the preincubation and injection, the DM assisted peptide binding was abolished. Therefore, SEA is able to block the DM function in this assay.

One possible explanation for blocking of DM function by SEA is that DM and SEA bind to the same site on DR1. To test this interpretation, other Superantigens such as SEB and TSST-1 were used. SEB and TSST-1 only bind DR1 through the site on the conserved α chain. Neither SEB, nor TSST-1 blocked the DM function. Hence, if the SEA and DM bind to the same site on DR1, it would be through the Zinc mediated binding site at H81 that is also located on the helix close to pocket 1.

EDTA Restores the DM Function in the Presence of SEA

Since the SEA blocking effect for DM is dependent on the zinc mediated binding site, the effect of EDTA(ethylenediamine-tetra-acetic acid) on SEA blocking was tested by including 50 mM EDTA in the sample solution before the samples were injection over the peptide surfaces. EDTA is a

known metal ion-chelating agent, which will deplete the metal ions in the solution (e.g. Zinc ion). As shown in Fig. 7, SEA abolished the DM assisted peptide binding in real time. In the presence of EDTA, SEA is not able to bind DR1 through the Zinc mediated binding, and therefore, when the sample is injected over the peptide surface, the DM assisted peptide binding should be restored. Indeed, as shown, 50 mM EDTA restores the DM assisted peptide binding of wtDR1 to $HA_{Anchorless}$ and HA305–318 surfaces. The level of binding is even higher than that in the absence of SEA possibly because of larger molecular masses composed of SEA bound to wtDR1. Again, this data strongly suggests that the SEA blocks the DM function through its zinc mediated binding site.

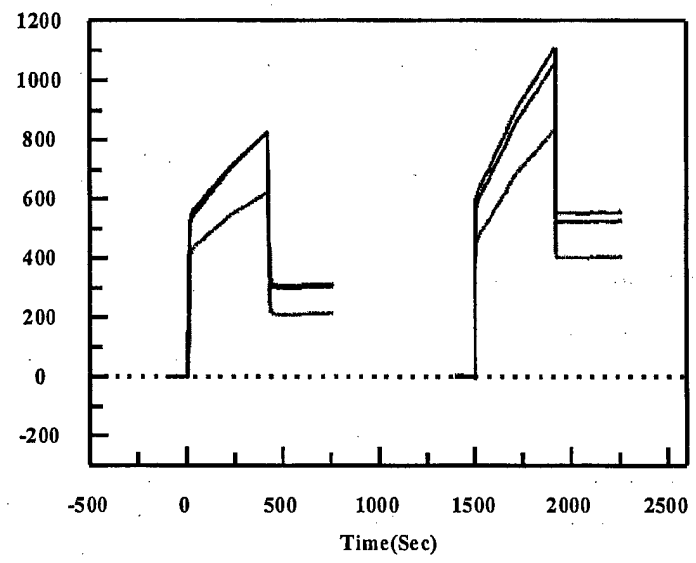

Figure 8. **The effects of SEB and TSST-1 on the DM assisted peptide binding in real time.** 2.4 μM wtDR1 in complex with HA_{Y308A} peptide was isolated by the procedure described in the legend to Fig 4, and then was incubated at 37°C with or without 7 μM SEB or TSST-1 in the presence of 1 μM DM for 20 min. Samples (citrate buffer, pH 6.0) were injected over two different peptide surfaces (Cys-HA, and $Cys\text{-}HA_{Anchorless}$) of BIAcore 2000 at a flow rate of 4 μl/min for 7 min followed by washout (citrate buffer plus 0.01% Tween 20, pH 6.0) at a flow rate of 5 μl/min. Sensograms of DR1 binding are shown in different colors: in the presence of DM (red), in the presence of SEB and DM (blue), and in the presence of both DM and TSST-1 (black).

SEB, TSST-1, or mutant SEA do not Blocks peptide dissociation of wt DR1 complexes

Another possible explanation for the SEA blocking effect is that instead of competing with DM binding, SEA changes the conformation of DR1/peptide complex to a stable conformation which is no longer the substrate of DM. To test the hypothesis, the dissociation rate of FITC-labeled peptide from wt DR1 complexes was determined in the presence of SEA, SEA mutant, SEB, and TSST-1 at 37 °C in PBS. Indeed, the presence of SEA dramatically increased the half-life of the wt DR1/ $FITC\text{–}HA_{Anchorless}$ from 1.7 hrs to 100hrs in solution. Whereas, SEB, SEA mutant, and TSST-1 had no significant effects on the dissociation rate for $FITC\text{–}HA_{Anchorless}$ from wtDR1 molecules (**Fig. 8**). These observations clearly demonstrated that SEA converts the short-lived peptide/DR1 complexes into stable complexes either by changing the conformation of the complexes or just simply by physical blocking of the dissociation of peptide from the DR1 molecules. Moreover, the presence of 50 mM EDTA abolished the effect of SEA suggesting that the Zinc dependent binding site is involved.

SEA Blocks the Peptide Binding of Mutant $DR1_{\beta G86Y}$ in Real Time

In the peptide dissociation assay, we have shown that SEA converts the short-lived peptide/DR1 complexes into stable complexes either by changing the conformation of the complexes, or by physical blocking of peptide dissociation from the DR1 molecules. To investigate the latter

possibility, the peptide binding of mutant $DR1_{\beta G86Y}$ in real time was measured in the presence of SEA. We observed that mutant $DR1_{\beta G86Y}$ is able to bind to the $HA_{Anchorless}$ without any help because it binds peptide more efficiently and stays in receptive form once the peptide is dissociated. If SEA was preincubated with mutant $DR1_{\beta G86Y}$ and was present in the sample mix during the injection, no binding was observed. In the presence of 50 mM EDTA, the peptide binding is restored. These results again indicated the SEA either changes the short-live peptide/DR1 complex into stable complexes or simply blocks release of the peptide from DR1 groove.

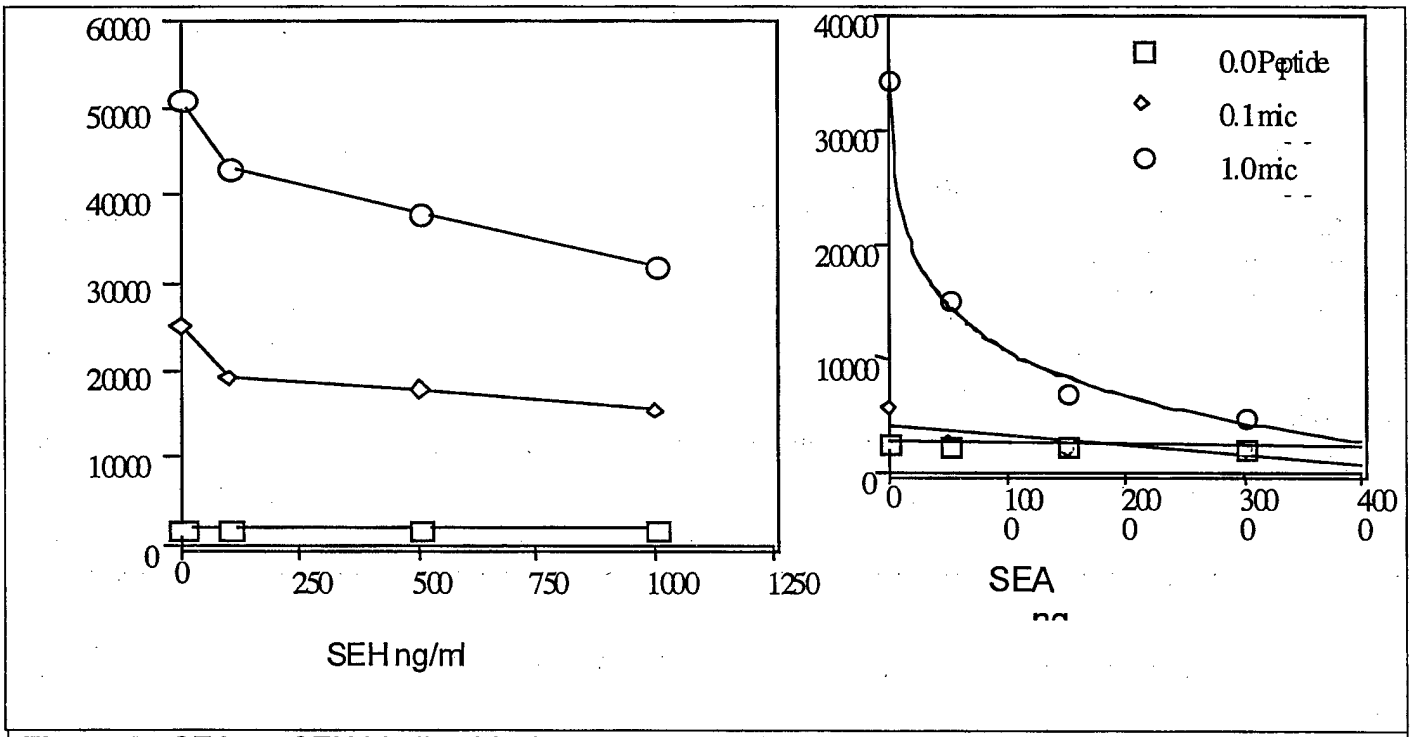

Figure 9. SEA, or SEH binding blocks recognition of peptide-DR1 by T cells. B cells expressing HLA-DR1 were pulsed with 0, 0.1, or 1mM HA306-318 peptide for 3 hours. Unbound peptide was removed by washing and then SEH or SEA was added at concentrations shown on the x-axis. T cells were incubated with the B cells for three days. 3HTdr was added 12 hours before the cells were harvested and counted for 3H incorporation (y-axis).

SEA, or SEH binding blocks recognition of peptide-DR1 by T cells

Binding of SEA to MHC class II has been a subject of investigations for the past two decades. SEA has two binding sites on class II, a low affinity alpha chain binding-site and a high affinity beta chain. Because of these two binding sites, it has been hypothesized that SEA cross-links

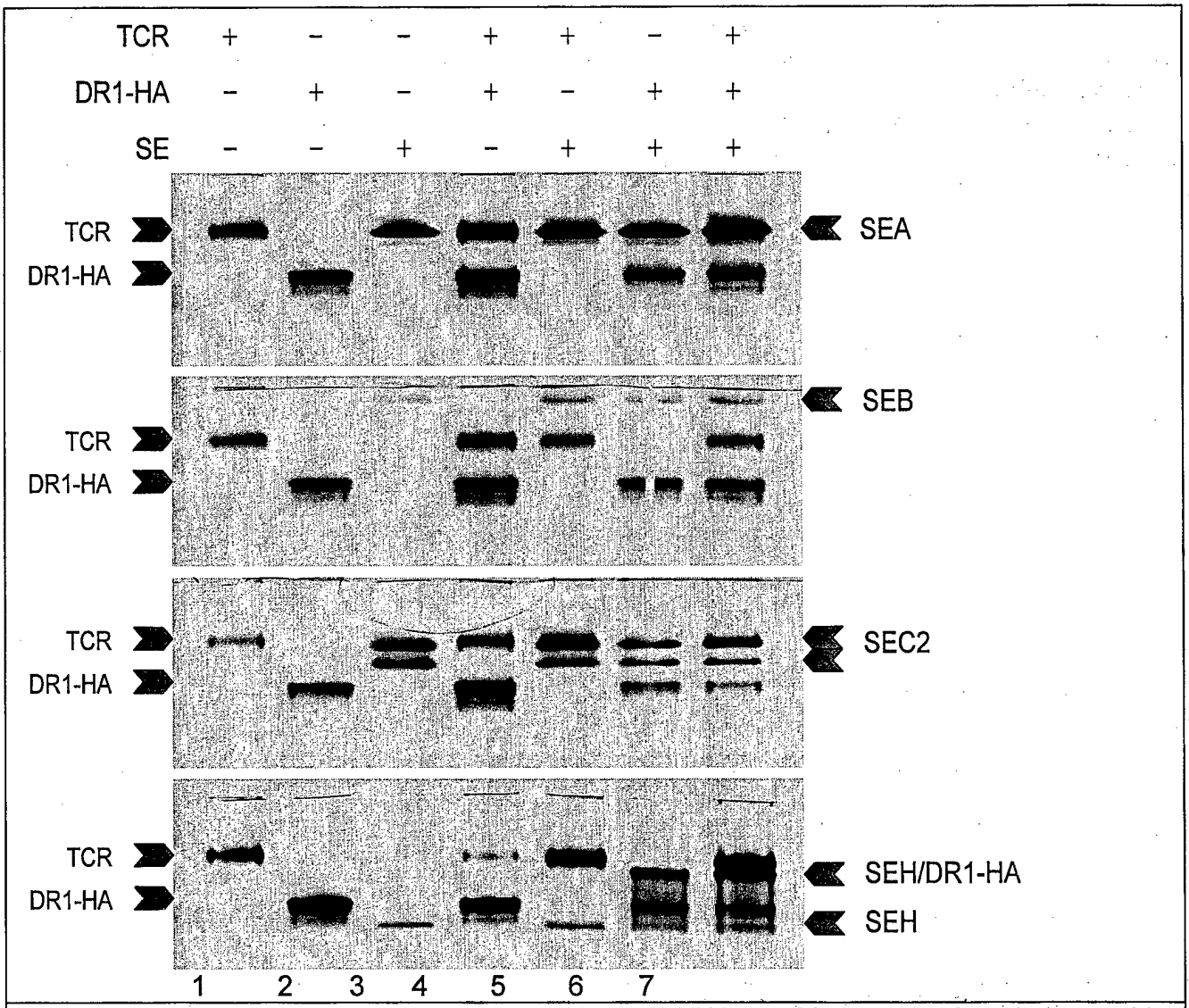

Figure 10. CI -1 soluble TCR interacts with HLA-DR1/HA peptide complex in solution and is detectable by a native PAGE gel. The Native-PAGE gel above demonstrates a single band for the TCR that interacts well with SEH (the lowest panel). Due to restrictions intrinsic to the native gels a new band depicting a ternary complex between TCR and DR1-HA peptide is not visible. However, reduction in DR1, and in TCR band densities when DR1 and TCR are mixed together suggests formation of ternary complexes. Interestingly, inclusion of SEH inhibits ternary complex formation as shown by disappearance of DR1-HA band but presence of TCR band when all three components are mixed (last lane). It is noteworthy that SEH binding to DR1 generates a new band.

two class II molecules. To better understand the SEA interaction with DR molecule, we revisited this question using purified HLA-DR1, SEA, and other SAgs, peptides binding to DR1, and purified soluble HLA-DM. We investigated the effects of SEA binding on the kinetics of binding and dissociation of peptides that form short-lived complexes with HLA-DR1.

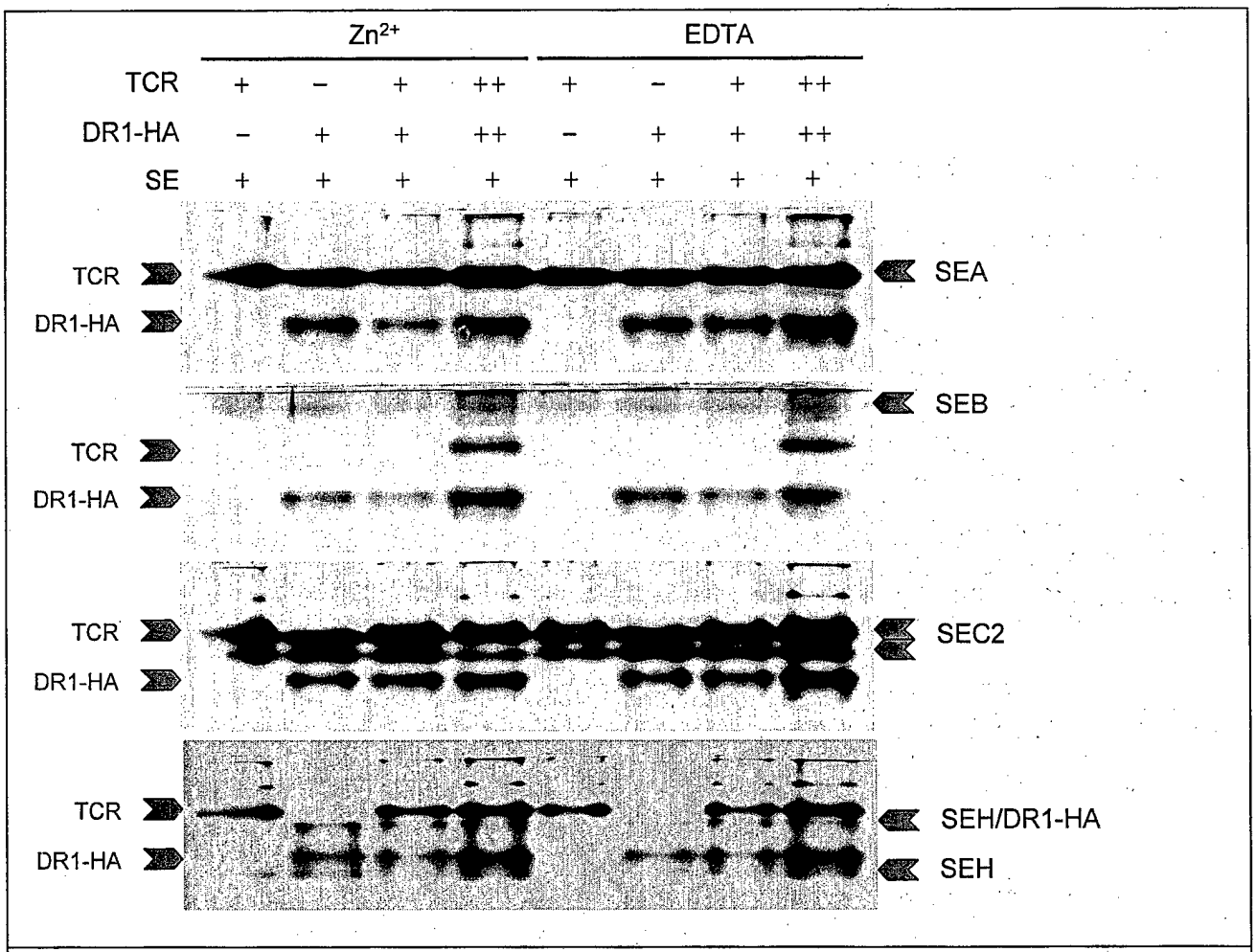

Figure 11. Chelating Zn++ inhibits formation of DR1/SEA and DR1/SEH complexes as well as ternary complexes with TCR. 400 ng each of SAG, DR1/P and TCR were incubated for 30 min at 37°C in PBS + Zn++ with or without EDTA. The last lanes in each set include double concentration of the protein. Samples were then mixed with native sample buffer (no SDS, or reducing agents and samples were not boiled) and subsequently gels were Silver-stained. As shown, a new band appears upon binding of SEH to DR1-HA, which is diminished in the presence of EDTA. Also, SEA binds to

The experiments shown in **Fig. 9** demonstrate complete inhibition of DR1/P recognition by CL-1 T Cells in the presence of SEA and moderate inhibition in the presence of SEH. These observations further support the notion that SEA binds to DR1 is through both α and β chains of DR and occurs at a stochiometry of 1:1. Thus, our experiments favor a 1:1 binding stochiometry for the SEA to DR1 for the following reasons: a) In the dissociation kinetics experiments, SEA binding to low affinity wt DR1/$HA_{Anchorless}$ complex dramatically increases the half-life of the peptide from a mere 1-2 hours to days. b), preincubation with SEA prevented the real time binding of mutant $DR1_{\beta G86Y}$ to the peptide surface which is in favor of the model that SEA may block the generation of empty peptide receptive conformation in the sample by forming a bridge across the peptide binding groove. c) EDTA that disrupts the zinc-mediated peptide binding does not interfere with peptide dissociation, d) a mutant SEA

molecule that has disrupted b chain binding site also does not interfere with peptide dissociation or DM acceleration of peptide release. e) SEA binding to DR1/$HA_{306-318}$ peptide complexes interferes with antigen recognition by CL-1 T cells by inhibiting contacts between T-cell receptor and MHC molecule.

The recent X-ray crystal structures of SpeC bound to HLA-DR2a (Sundberg et al., 2002a), and SEH in complex with HLA-DR1(Sundberg et al., 2002b), are both in favor of our findings. SpeC and SEH bind to the high-affinity, zinc-dependent site on the MHC class II molecules. In both structures, the SAgs make extensive contacts with the peptides, such that peptide accounts for almost one third of the MHC surface in contact with the TCR, similar to the TCR-peptide/MHC ternary complexes. Thus, it is reasonable to imagine that this extensive interaction may impose conformational constrains on the peptide/MHC complex and inhibit dissociation of the bound peptide, a process that requires conformational changes (Chou and Sadegh-Nasseri, 2000; Natarajan et al., 1999a; Natarajan et al., 1999b; Sadegh-Nasseri, 1994; Sadegh-Nasseri and Germain, 1991; Sadegh-Nasseri and Germain, 1992; Sadegh-Nasseri and McConnell, 1989; Sadegh-Nasseri et al., 1994). In agreement with this interpretation, HLA-DM failed to generate an open or peptide-receptive conformation on SEA or SEH bound DR1 molecules. Interestingly, binding of SEA to the α chain of DR as it occurs in the presence of EDTA does not affect DM functions. Additionally, SEB, or TSST-1 that bind to the α chain only also is not inhibitory to DM functions. Thus, altogether, we have demonstrated that binding of SAGs such as SEA or SEH to DR molecules constrains the MHC II making to become refractory to conformational changes necessary for peptide association and dissociation.

TCR reactivity with Superantigens

In previous sections I reported design and construction of CL-1 TCR and the analyses if its purity. In the following section I would focus on the molecular interaction of TCR with HLA-DR1 and SAGs.

In the experiments shown in Fig 10, we tested purified TCR protein for reactivity with SAGs and DR1/P by Native-PAGE. In lanes 1-3, components of the system are shown individually, and lanes 4-7 depict combinations of two and three. Superantigens, SEA, SEB, SEC-2 and SEH in combination with HLA-DR1-HA peptide and /or purified TCR + SAG are shown. TCR, HLA-DR, SEA, SEB, and SEH all migrate as clean single bands. However, SEC-2 migrates as two species. Formation of SEC2/DR1/TCR complex is implied upon a decrease in DR1 band in Fig 10 lane 7.

A new band is formed migrating slower than DR1/P alone when SEH and DR1 are mixed (lane 6) consistent with solution binding experiments in Fig. 6. However, complexes of SEA/DR1 do not migrate as a distinct band in the Native PAGE under the conditions used here. Neither ternary complex between TCR and DR1-HA peptide is visible by silver staining. However, reduction in DR1 and in TCR band densities when DR1 and TCR are mixed together suggests formation of ternary complexes. Interestingly, inclusion of SEH inhibits ternary complex formation as shown by disappearance of DR1-HA band but presence of TCR band when all three components are mixed (last lane). SEC2 was shown to have stimulatory effects on Cl-1 T cells. Lane 7 in SEC2 panel

shows a significant reduction in DR1/P band in the presence of TCR and SEC2, implying formation of quaternary complexes of TCR/DR1/P/SEC2.

Because of the EDTA effects of inhibition of the DR1 beta chain binding we repeated these experiment in two sets. In one set, Fig 11 left lanes, excess Zn++ was included in the sample mix to optimize interactions of SEA or SEH with DR1. In the right lanes, 10mM EDTA was included for depletion of Zn++. As shown, reductions in the level of DR1 band is seen when SEA Binds DR1 (lane 3). The reduction is not seen when the samples include EDTA (lane 7).

Thus, by use of Native-PAGE, and silver staining, ternary and quaternary complexes of TCR with ligands are not visible by silver staining (Figure 4a). To detect formation of the ternary complexes we used western blotting in Fig 4B. Figure 4a and 4b represent identical samples detected by Silver stain in A, or subjected to Western blotting followed by staining with a polyclonal antibody raised against sHLA-DR1 in rabbits. As shown, the Ab shows no reactivity with the TCR alone (4B lanes 1 and 10) but as expected, it stains DR1/peptide protein as a single band. The antibody stains a new band as well that increases proportionately as the ratio of DR1:TCR increases from 1:1 to 1:3. As TCR alone does not have reactivity with this antibody, this band represent a ternary complex Interestingly, when TCR is added at different ratios, a new band appears that migrates slower and increases upon increasing the ratio of TCR:DR1 from 1:1 to 3:1. This new band, we think, is the TCR-DR1-peptide ternary complex that migrates at the exact location as the TCR alone in the Native-PAGE!

KEY RESEARCH ACCOMPLISHMENTS

A. TCR cloning in bacterial expression system;

- Beta chain of TCR was produced in inclusion bodies of the expressing bacteria to over 90 % purity

- Alpha chain of TCR was produced in inclusion bodies but mixed with many other proteins

- We properly folded TCR from bacteria and confirmed its conformation by Ab recognition in an ELISA. Unfortunately, yield was quite low. We attempted to optimize refolding of TCR by; a) mixing a larger proportion of a chain protein with the beta chain, b) try several protein folding methods, and c) use other strains of bacteria expressing the gene to increase compatibility with the alpha chain. Adding 5% glycerol helped refolding but not high enough to ensure sufficient quantities for large number of experiments.

B. TCR cloning in bacculovirus expression system and experimental findings

- We have successfully cloned a human TCR, CL-1, in bacculovirus expression system.

- We have affinity purified the protein to over 95%purity and have shown reactivity with its natural ligands, HLA-DR1 in complex with specific peptides, and SAGs.

- We have shown that CL-1 TCR is reactive with SEC2, and to a lesser extent to SEB.

- We have established that SEA and SEH interact with HLA-DR1

- The SEA and SEH binding to HLA-DR1 interferes with CL-1 recognition of peptide/DR.

C. Superantigen interaction with HLA-DR1

- We have demonstrated that SEA binds HLA-DR1 through both α and β chains.

- In the dissociation kinetics experiments, SEA binding to low affinity wt DR1/$HA_{Anchorless}$ complex dramatically increases the half-life of the peptide from a mere 1-2 hours to days.

- Preincubation with SEA prevented the real time binding of mutant $DR1_{\beta G86Y}$ to the peptide surface which is in favor of the model that SEA may block the generation of empty peptide receptive conformation in the sample by forming a bridge across the peptide binding groove.

- EDTA that disrupts the zinc-mediated peptide binding does not interfere with peptide dissociation,

- A mutant SEA molecule that has disrupted b chain binding site also does not interfere with peptide dissociation or DM acceleration of peptide release.

- SEA binding to DR1/$HA_{306-318}$ peptide complexes interferes with antigen recognition by CL-1 T cells by inhibiting contacts between T-cell receptor and MHC molecule.

REPORTABLE OUTCOMES

1) Chou, C-L, Mirshahidi, S, Khoruzhenko, S. & **S. Sadegh-Nasseri.** SEA binds to a single HLA-DR1 via two sites and inhibits peptide dissociation and specific T cell recognition. 2004 (manuscript in preparation).

2) Chou, C-L, Ph.D. Thesis, 2003. Graduate Program in Biophysics, Johns Hopkins University

CONCLUSIONS

Our findings present insights into the biochemistry of TCR interaction with SAGs that change the conventional understanding of Superantigen interactions with the TCR from a merely bridging the MHC class II with the TCR Vβ lateral surface to a novel interaction that involves the entire MHC II groove.

Moreover, our findings regarding SEA interaction with HLA-DR1 at a stochiometry of 1:1 opens ways for new approaches in design of vaccines against SEA toxicity.

List of personnel who contributed to this work and received salary support for different periods of time during the award:

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Saied Mirshahidi, Ph.D. Postdoctoral Fellow
Sarat Dalai, Ph.D. Postdoctoral Fellow
Stanislav Khoruzhenko, Technician

Personnel who contributed to this work and did not receive salary support:

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Chih-Ling Chou, Graduate Student in Program in Biophysics at the time, Ph. D. (May 2003)
Isamu Hartman, Graduate Student in Program in Immunology
Kedar Narayan, Graduate Student in Program in Immunology
Yu Li, Graduate Student in Program in Immunology

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